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Determination of Free Fatty Acids in Fat

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▶ **Naturally occurring free fatty acids may be determined in the presence of large amounts of unsaponified fat by adsorbing the free fatty acids on a strong anion base exchange resin, washing the resin free of fat with petroleum ether, and converting the free fatty acids directly on the resin to their methyl esters with anhydrous methanol–hydrochloric acid. The nature and concentration of the fatty acids are determined by gas chromatography.**

Several studies in this laboratory are concerned with the nature and concentration of free fatty acids present in cured and cooked meats and with their possible effect on flavor. This work describes a method for the separation of the free fatty acids from unsaponified fat by adsorption of the fatty acids on a strong anion base exchange resin, converting the fatty acids to their methyl esters without prior elution from the adsorbent, and the eventual separation of these esters by gas chromatography on a poly(vinyl acetate) column. The fatty acids present are quantitatively estimated, using *n*-heptadecanoic acid as an internal standard.

[1] Deceased.

METHOD

Reagents. Amberlite IRA-400 (Rohm & Haas Co., Philadelphia, Pa.).

Heptadecanoic acid (Eastman Kodak, Rochester, N. Y.) recrystallized twice from methanol.

Methanol–hydrochloric acid, anhydrous, 5 to 10% acid.

Poly(vinyl acetate), grade AYAC (Bakelite Division, Union Carbide Corp., New York).

Chromosorb R, 30- to 60- mesh (Celite Division, Johns-Manville, New York).

Fatty acid methyl esters (Hormel Foundation, Austin, Minn.).

Apparatus. GC-2 gas chromatograph, equipped with four-filament thermal conductivity detector (Beckman Instrument Co., Fullerton, Calif.).

Recorder, 1 mv. (Brown Instrument Division, Minneapolis-Honeywell, Philadelphia, Pa.).

Pretreatment of Resin. Ten grams of Amberlite IRA-400 are stirred with 25 ml. of 1*N* sodium hydroxide for 5 minutes. The resin is allowed to settle and the supernatant liquid is discarded. The resin is successively washed with several portions of distilled water to remove free alkali, then with three 25-ml. portions of anhydrous ethyl alcohol to remove water, and finally with three 25-ml. portions of petroleum ether to displace the ethyl alcohol.

Separation of Fatty Acids from Fat. If fat emulsions are analyzed, a volume of emulsion containing 0.1 to 1.0 gram of fat is diluted in a 125-ml. separatory funnel with an equal volume of ethyl alcohol and made acid to bromophenol blue with several drops of 1*N* sulfuric acid. The mixture is extracted with two 20-ml. portions of petroleum

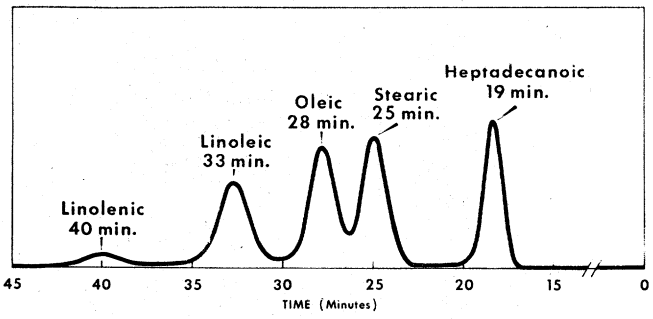

Figure 1. Separation of C_{18} fatty acids and C_{17} internal standard on PVA-packed column

ether and the aqueous layer discarded (*1*). For unemulsified fat samples, 0.1 to 1.0 gram is dissolved directly in petroleum ether.

In either case 10 to 25 mg., accurately weighed, of *n*-heptadecanoic acid are added to the petroleum ether solution, which is decanted onto 10 grams of the pretreated resin in a 250-ml. Erlenmeyer flask. Two 5-ml. portions of petroleum ether are used to wash the residual solution into the flask. The solution, plus resin, is stirred (magnetic stirrer) for 5 minutes. The resin, plus adsorbed fatty acids, is allowed to settle and the supernatant liquid discarded. The residue is then washed free of fat by stirring with three successive 25-ml. portions of petroleum ether and discarding the washings.

Preparation of Methyl Esters. The adsorbed fatty acids are converted to the esters directly on the resin. A 25-ml. portion of anhydrous methanol–hydrochloric acid is added to the resin and the mixture stirred for 25 minutes. The methanol solution is decanted through a rapid filter paper into a 250-ml. separatory funnel. The resin is washed by stirring for 5 minutes with two successive 15-ml. portions of anhydrous methanol–hydrochloric acid, again decanting each wash through the filter. Then 10 ml. of distilled water are added to the combined methanol extracts and the solution is extracted with 50 ml. of petroleum ether. The aqueous phase is drained into a second separatory funnel and extracted twice with 20-ml. portions of petroleum ether. The combined petroleum ether extract is washed with 50-ml. portions of water until free of acid, dried over anhydrous sodium sulfate, and then concentrated by a stream of dry nitrogen on a steam bath. The fatty acid esters are quantitatively transferred with petroleum ether to a 1-ml. volumetric flask; an aliquot is then used for the gas chromatographic separation.

Gas Chromatographic Separation. The partitioning medium is a poly(vinyl acetate) of approximately 1500 molecular weight (vinylite AYAC) in a ratio of 1 to 5 w./w. on Chromosorb 30- to 60-mesh (*4*). The column packing is prepared by slurrying the Chromosorb with the required amount of a 20% solution of the polymer dissolved in acetone. Additional acetone may be necessary to form a uniform slurry. The slurry is spread out in an enameled or stainless steel pan and the acetone evaporated at room temperature. The Chromosorb is stirred occasionally, and the volatiles are removed from the packing by heating in a vacuum oven at 130° C. for 1 hour. An 8-foot coiled copper column ($^1/_4$ inch in outside diameter and 0.03-inch wall thickness), packed with the poly(vinyl acetate) packing, is prepared for use with the Beckman GC-2. The column, with helium flowing through, is preconditioned by heating for several hours at the operating temperature of 205° C. The helium flow rate is 80 to 85 ml. per minute as measured at room temperature by a soap bubble flowmeter at the column exit. The column and detector cell temperatures are maintained at 205° C. The pressure drop along the column is about 15 p.s.i.g. The sample injected into the gas stream is generally 5 μl. of a petroleum ether solution containing 250 to 1000 γ of the esters being studied.

Quantitative Analysis of Methyl Esters. The acids of primary interest in this study include lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic, and the added internal standard, *n*-heptadecanoic acid. A standard solution in petroleum ether containing 5 mg. per ml. of each of the pure esters is prepared and 5, 10, and 20 μl. are chromatographed. The width at the base of the peak is measured between the base-line intercepts of the tangents to the peak; the peak area is taken as one half peak width times peak height (*5*). The weight of ester represented by 1 sq. mm. was equal within $\pm 1.0\%$ for each of the three dilutions for a given ester. The different fatty acid esters do not give the same area response per microgram, and so the areas are calibrated for each acid in micrograms of acid per square milliliter. When known mixtures of fatty acids in fat are carried through the procedure and appropriate aliquots chromatographed, quantitative results are obtained by using a factor based on the per cent recovery of the added *n*-heptadecanoic acid. Thus, if in a given run the over-all recovery of the C_{17} acid is 82%, then all other acid recoveries are divided by 0.82 to give the corrected recovery values. Absolute recoveries generally range from 60 to 95%.

RESULTS AND DISCUSSION

The separation of the fat from the isolated fatty acids must be complete. If any fat is carried over to the esterification stage, trans-esterification can take place and high results will be obtained for the free fatty acids. Preliminary attempts to separate the free fatty acid completely from the fat by partitioning between solvent pairs were not successful, while adsorption of the fatty acids on a strong anion base exchange resin was simple and gave a clean-cut separation of the acids from the fat.

The use of *n*-heptadecanoic acid as an internal standard is based on the following assumptions: that this compound is usually absent, or present below the level of detection by this method, in naturally occurring fats; that the fatty acids, including the added C_{17} acid, will be adsorbed completely (or to an equal extent) by the anion exchanger; that the esterification will proceed in similar fashion for all of the acids. These are not unreasonable suppositions, since the pK's for these acids are all of the same magnitude, and their validity is confirmed by the recovery data reported in Table I for a known mixture of fatty acids covering a range of concentration similar in ratio to that found in lard. The data in Table I are corrected for small amounts (less than 1 mg. of any given fatty acid) of free fatty acids originally present in the fat. These

Table I. Recovery Data for Free Fatty Acid Mixture Added to 10 Ml. of 5% Fat Emulsion[a]

Acid	Milligrams Added	Milligrams Recovered[b]	Recovery Corrected, mg.[c]	Recovery %
Lauric	0.44	0.12	0.18	40.9
Myristic	4.10	2.74	4.11	100.2
Palmitic	24.00	16.32	24.48	102.0
Stearic	10.50	7.06	10.60	100.9
Oleic	35.40	23.66	35.50	100.3
Linoleic	2.20	2.13	3.20	145.4
Linolenic	0.40	0.47	0.69	172.5
Total	77.04	52.50	78.76	102.2

[a] Fatty acids converted to their methyl esters and a 5-μl. aliquot in petroleum ether containing approximately 385 γ of total fatty acids chromatographed over poly(vinyl acetate) at 205° C.
[b] Corrected for free fatty acids present in fat blank.
[c] Based on 66.6% recovery of added *n*-heptadecanoic acid.

blanks were determined by adding a known amount of *n*-heptadecanoic acid to the fat and analyzing for fatty acids as described. In this particular sample, the amounts of lauric, linoleic, and linolenic acids actually chromatographed were approximately 2, 10, and 2 γ, respectively. The peak areas for these small amounts of acid are difficult to measure, and the results are erratic using a packed column and a thermal conductivity detector. For samples of 25 to 200 γ of free fatty acids the recoveries as shown are excellent.

Absolute recoveries generally run from 60 to 95%; quantitative results without the use of an internal standard would be unobtainable. The recrystallized C_{17} acid used as the internal standard, when carried through the esterification procedure and chromatographed, yielded only a trace of impurity at a position corresponding to the C_{18} saturated acid. None of the fat samples analyzed in this study has shown a peak at this position.

Figure 1 shows the chromatogram for the separation of the C_{17} standard and the C_{18} fatty acids. The retention time for methyl palmitate is 12 minutes and its separation from the C_{17} acid is also complete. The poly(vinyl acetate) (PVA) used as the partitioning medium is preferred to the polyester-type materials that have been reported (*2*, *6*, *7*). PVA is readily available commercially and does not show excessive "bleeding." The products of the thermal degradation of PVA are acetic acid and polyvinylacetylene (*3*), and in preliminary stability studies at 205° C. only a slight initial loss of acetic acid (0.5 mg. per hour) was found. Thus, if the separated fatty acid methyl esters are collected for spectrophotometric study, one can readily compensate for traces of acetic acid collected with the sample.

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